Pestic. Sci. 1996, **47**, 185–190

Chlorotoluron Metabolism in Leaves of Resistant and Susceptible Biotypes of the Grass Weed *Alopecurus myosuroides*

Ralph J. Hyde,[a]* David L. Hallahan[b] & John R. Bowyer[a]‡

[a] Division of Biochemistry, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK
[b] Department of Biochemistry and Physiology, IACR-Rothamsted, Harpenden, Herts AL5 2JQ, UK

(Received 19 October 1995; accepted 22 January 1996)

Abstract: The metabolism of the herbicide chlorotoluron by susceptible and resistant biotypes of the grass weed, *Alopecurus myosuroides*, was examined. After administration of radiolabelled herbicide to leaves, metabolites were extracted and analysed. The metabolites identified consisted of mono-demethylated-, di-demethylated- and ring methyl-hydroxylated chlorotoluron. Metabolism was more extensive in the resistant biotype, yielding principally the non-phytotoxic ring methyl-hydroxylated metabolite. The metabolites observed are characteristic of the activity of cytochrome P450 mixed-function oxygenase action. The specific cytochrome P450 inhibitor, 1-aminobenzotriazole, reduced accumulation of the ring methyl-hydroxylated metabolite in the resistant biotype.

Keywords: chlorotoluron, *Alopecurus myosuroides*, cytochrome P450, herbicide metabolism.

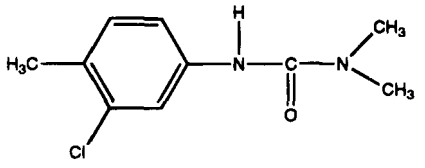
1 INTRODUCTION

In recent years, the severe selection pressure imposed by repeated applications of herbicides with similar modes of action has resulted in the appearance of resistant biotypes of previously sensitive weed species.[1] *Alopecurus myosuroides* Huds. (black-grass) is a common weed in cereal crops throughout much of Western Europe. In England, a biotype (Peldon A1) from Peldon, Essex, was identified which was highly resistant to the phenylurea herbicide, chlorotoluron[2,3] (Fig. 1, **1**), and cross-resistant to a number of herbicides with different modes of action.[2–4]

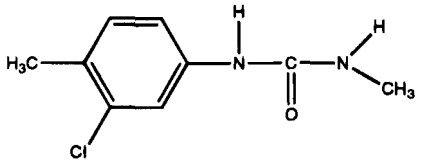
Although more than one mechanism may be involved in cross-resistance in biotypes of *A. myosuroides*, resistance to chlorotoluron in biotype Peldon has been attributed to enhanced metabolism.[5,6] In tolerant cereal crops such as wheat and maize, chlorotoluron is rapidly metabolised to non-toxic compounds.[7–9] The reactions involved, ring methyl-hydroxylation and *N*-demethylation, are characteristic of cytochrome P450 monooxygenase action.[10,11] Both these reactions are inhibited by treatment of plants with the specific cytochrome P450 suicide substrate 1-aminobenzotriazole (1-ABT), although *N*-demethylation is less sensitive than ring methyl-hydroxylation.[9,12] 1-ABT also increased phytotoxicity of chlorotoluron in resistant *Lolium rigidum* Gaud., and was shown to inhibit *N*-demethylation in this plant.[13] A specific involvement of cytochrome(s) P450 in chlorotoluron metabolism was confirmed by studies with isolated membrane fractions of wheat and maize, where ring methyl-hydroxylation was conclusively shown to be due to monooxygenase activity.[14,15] In *A. myosuroides*, it was shown that susceptible plants were capable of metabolising chlorotoluron and other herbicides.[16] Resistant (Peldon) plants were shown to metabolise chlorotoluron more extensively to conjugated products than the sensitive

* Current address: Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT.
‡ To whom correspondence should be addressed.

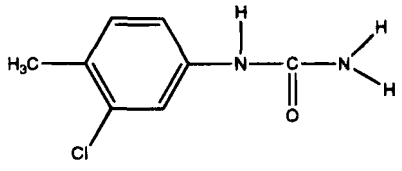
Pestic. Sci. 0031-613X/96/$09.00



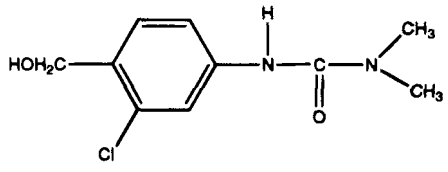
Chlorotoluron (1)



Mono-demethylated chlorotoluron (2)
CGA 16339



Di-demethylated chlorotoluron (3)
CGA 16340



Ring methyl-hydroxylated chlorotoluron (4)
CGA 18029

Fig. 1. Structures of compounds discussed in text.

(Rothamsted) biotype.[17] 1-ABT has been shown to synergise chlorotoluron activity against resistant (Peldon) *A. myosuroides*,[5] and to inhibit metabolism of the herbicide to conjugated products,[17] but the identity of the metabolites in the conjugated products was not reported, and no unconjugated metabolites were described. Although somewhat circumstantial, these results suggest (by analogy with the studies on wheat and maize) that enhanced metabolism might be responsible for chlorotoluron resistance in *A. myosuroides* (Peldon). The effects of 1-ABT on herbicidal efficacy and metabolism further suggest that cytochrome P450 monooxygenase(s) may play a role in this metabolism. To date, however, little information on the nature of the metabolites produced in *A. myosuroides* from chlorotoluron has been presented. We show here that in *A. myosuroides*, chlorotoluron is metabolised to monodemethylated (Fig. 1; **2**), di-demethylated (**3**) and ring methyl-hydroxylated (**4**) metabolites characteristic of the action of cytochromes P450 in other plants. Metabolism in both susceptible (Rothamsted) and resistant (Peldon) biotypes is examined, and an effect of the specific cytochrome P-450 inhibitor 1-ABT on the metabolite profile is demonstrated.

2 MATERIALS AND METHODS

2.1 Plant growth

Seed of *A. myosuroides* was collected from winter wheat fields in England at Rothamsted (Hertfordshire) and Peldon (Essex), in 1990, and was supplied by Dr S. R. Moss (Crop Management Department, IACR-Rothamsted). Seed was germinated on filter paper soaked with potassium nitrate (2 g litre^{-1}) and seedlings maintained in Kettering loam:grit (5:1) in a constant environment chamber (14 h day, 16°C, RH 70%; 10 h night, 12°C, RH 80%).

2.2 Source of chemicals

[^{14}C]Chlorotoluron (^{14}C-carboxyl; 1.99 GBq mmol^{-1}) was obtained from Zeneca Agrochemicals (Jealotts Hill, UK). Chlorotoluron metabolite standards were obtained from Dr W. J. Owen (Biochemistry Department, Royal Holloway, University of London). 1-Aminobenzotriazole was obtained from Sigma.

2.3 Chemical treatment

[^{14}C]Chlorotoluron (8·33 kBq) was administered to detached leaves of 14-day-old *A. myosuroides* by standing leaves in 10 ml of herbicide (27 μM) in one-third strength modified Hoagland's solution[18] in a 20-ml bottle. Treatments were carried out for 24 h in a constant environment chamber (conditions as above); after this period, leaves were transferred to solution without herbicide for a further 24-h period. When required, 1-ABT (75 μM) was added to the solutions.

2.4 Extraction and analysis of metabolites

After treatment, leaves were washed with one-third strength modified Hoagland's solution (2 × 10 ml), frozen in liquid nitrogen, and stored at −70°C until required. The frozen leaves were ground in two aliquots (10 ml) of 80% methanol and the extract was centrifuged (10 000g_{av}, 10 min). Methanol was removed from the supernatant under vacuum. The aqueous residue remaining was extracted three times with dichloromethane, and the dichloromethane extracts dried over anhydrous magnesium sulfate. This fraction contained

parent herbicide together with unconjugated metabolites.

The aqueous residue was treated with β-glucosidase (1·0 mg ml^{-1} in 0.1 M sodium acetate, pH 4·4) for 16 h at 37°C, and then extracted with dichloromethane.

To the remaining aqueous residue, an equal volume of concentrated hydrochloric acid was added, and after 16 h at 50°C, the solution was extracted with dichloromethane.

Each extract was chromatographed on silica TLC plates with a 3-cm pre-absorbent strip (Whatman LK6DF) in saturated chloroform + ethanol + acetic acid (27 + 2 + 1, by volume). Radioactive bands were detected using a Berthold Tracemaster 2 linear analyser, scraped from the plates, and quantified by liquid scintillation counting. Metabolites were identified on TLC by co-chromatography with authentic compounds, which were visualised under UV light.

2.5 Microsome preparation and analysis

Leaves from 14-day-old plants were stored in liquid nitrogen until use. The leaves were homogenized using a mortar and pestle, in 200 ml $litre^{-1}$ glycerol, 5·0 mM dithiothreitol, 10 mM sodium ascorbate, 6.6 mg ml^{-1} leupeptin, 1·0 mM PMSF, 0·1 M MOPS, pH 7·2. The microsomal fraction was isolated by differential centrifugation, and carbon monoxide difference spectroscopy performed, as previously described.[19]

For determination of microsomal activity towards chlorotoluron, microsomes (0·25–0·4 mg protein) were incubated with [^{14}C]chlorotoluron (1·67 kBq; 500 μM) in 1·0 ml of a solution containing 200 ml $litre^{-1}$ glycerol, 6·7 mM glucose-6-phosphate, 2U glucose-6-phosphate dehydrogenase, 1·0 mM NADPH, 50 mM potassium phosphate, pH 7·4, for 25 min at 25°C. After stopping the reaction with hydrochloric acid (1 M; 1·0 ml), the mixture was extracted with dichloromethane (3 × 1·0 ml) the extract dried, and analysed by TLC as described above.

3 RESULTS AND DISCUSSION

The metabolism of chlorotoluron by *A. myosuroides* was assessed by feeding radiolabelled compound to excised leaves for a period of 48 h. After thorough washing, metabolites were extracted, analysed by TLC (after enzymatic and acid hydrolysis of conjugated metabolites), and quantified. The sum of the radioactivity in the initial methanol extract of the leaf tissue and that remaining in the herbicide solution after the incubation was routinely ≥95% of the radioactivity added. Metabolite identification was achieved by co-chromatography with authentic metabolite standards (Fig. 2). The data are presented in Table 1.

Even after enzymatic treatment and acid hydrolysis of the aqueous residue, significant amounts of radiolabelled herbicide metabolites remained unextracted into dichloromethane. Although we did not characterise this fraction further, it clearly represents a major detoxification route in these plants, and we assume it derives from the oxidised metabolites characterised below. The amount of radioactivity associated with this fraction was greater for resistant plants (55·8%) than for susceptible plants (26·2%), and indicates a more extensive overall metabolism of herbicide in resistant plants. This conclusion is further supported after analysis of the patterns of metabolites produced by the two biotypes.

In leaves of the resistant (Peldon) biotype, metabolism was extensive, with only 3·6% of the label recovered in the parent compound chlorotoluron (**1**) after 48 h of feeding. The remaining radioactivity recovered was distributed between mono-demethylated (**2**) (14·4%), di-demethylated (**3**) (4·7%) and ring methyl-hydroxylated (**4**) (21·5%) metabolites (Table 1). Analysis of metabolism in leaves from susceptible (Rothamsted) plants showed that these 'wild-type' plants were capable

TABLE 1
Metabolism of Chlorotoluron by Resistant (Peldon) and Susceptible (Rothamsted) Biotypes of *Alopecurus myosuroides*, and the Effect of 1-ABT

	Chlorotoluron (1)	*Mono-demethylated* (2)	*Di-demethylated* (3)	*Ring methyl-hydroxylated* (4)	*Non-hydrolysed material remaining in aqueous extract*
Rothamsted	21·7	31·7	12·7	7·7	26·2
Rothamsted + 1-ABT	37·0	28·5	9·8	8·6	16·0
Peldon	3·6	14·4	4·7	21·5	55·8
Peldon + 1-ABT	6·1	21·9	7·3	15·7	48·9

The radioactivity associated with each compound in the initial dichloromethane extract was added to that of each compound in the dichloromethane extracts after enzymic and acid hydrolysis. The radioactivity of each compound is then expressed as a percentage of the sum of the radioactivity of the compounds in the dichloromethane extracts and radioactivity remaining in the aqueous extract. The data presented are the average of two independent experiments in each case.

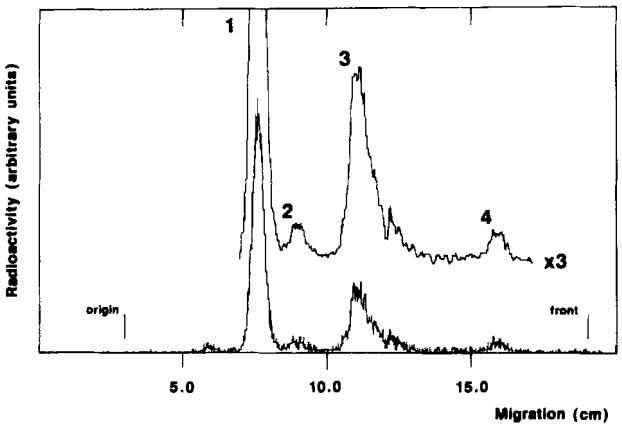


Fig. 2. Thin layer chromatography of extractable metabolites of chlorotoluron from resistant (Peldon) *Alopecurus myosuroides*. After chromatography, ^{14}C-labelled metabolites were located using a linear analyser. Metabolites were identified by co-chromatography with unlabelled reference compounds as ring methyl-hydroxylated chlorotoluron (**1**), di-methylated chlorotoluron (**2**), mono-demethylated chlorotoluron (**3**) and unmodified chlorotoluron (**4**).

of qualitatively similar metabolism to that found in the resistant biotype. Leaves from these plants also accumulated mono-dimethylated, di-demethylated and ring methyl-hydroxylated chlorotoluron metabolites (Table 1). However, metabolism in these plants was less extensive during the 48-h period, with 21·7% of herbicide remaining unmetabolised. In addition, metabolism in these plants favoured mono- and di-demethylation (31·7% and 12·7% of recovered radiolabel, respectively) over ring methyl-hydroxylation (7·7% of recovered radiolabel).

Thus, in the susceptible biotype, it would appear that the mono-demethylated metabolite, with 50–70% of the phytotoxicity of parent chlorotoluron,[7] accumulates rather than the non-phytotoxic ring methyl-hydroxylated metabolite (Fig. 3). The ring methyl-hydroxylated metabolite, on the other hand, is the major derivative produced in the resistant biotype (Fig. 3), which also accumulated more non-hydrolysable conjugates during the period of study. The accumulation of non-hydrolysable conjugates in the resistant biotype is probably a consequence of the increased metabolism to the ring methyl-hydroxylated derivative, since this compound is likely to be more amenable to conjugation.[20] Ring methyl-hydroxylation has been shown to be the principal metabolic pathway for chlorotoluron detoxification in wheat. Similar mechanisms to those which confer tolerance in cereal crops might therefore underly the resistance phenomenon in *A. myosuroides*.

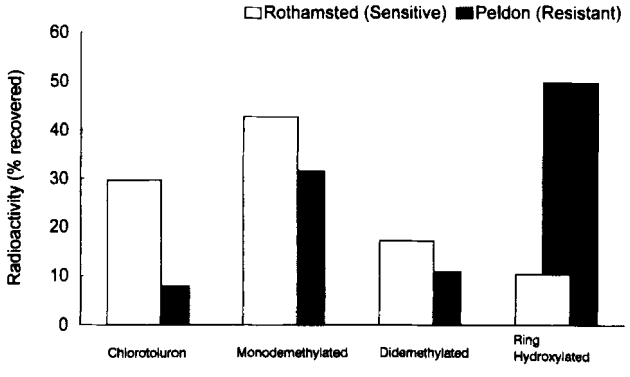


Fig. 3. Distribution of chlorotoluron metabolites following administration to susceptible (Rothamsted) and resistant (Peldon) *Alopecurus myosuroides*. The data are expressed as a percentage of the radioactivity recovered associated with identifiable compounds.

In chlorotoluron-tolerant wheat and maize plants, an involvement of microsomal cytochrome P450 enzymes in ring methyl-hydroxylation has been inferred from inhibitor studies.[9,12,14,21] In order to determine if similar enzymes are involved in metabolism in *A. myosuroides*, radiolabelled chlorotoluron was administered to *A. myosuroides* leaves in the presence of the specific cytochrome P450 inhibitor 1-ABT. The results are shown in Table 1 and Figure 4 and indicate differences in response to this inhibitor between the two biotypes.

With *A. myosuroides* Peldon, the addition of 1-ABT significantly inhibited production of the hydroxymethyl phenyl metabolite (Fig. 4A). Inhibition of ring methyl-hydroxylation by 1-ABT reduced overall detoxification

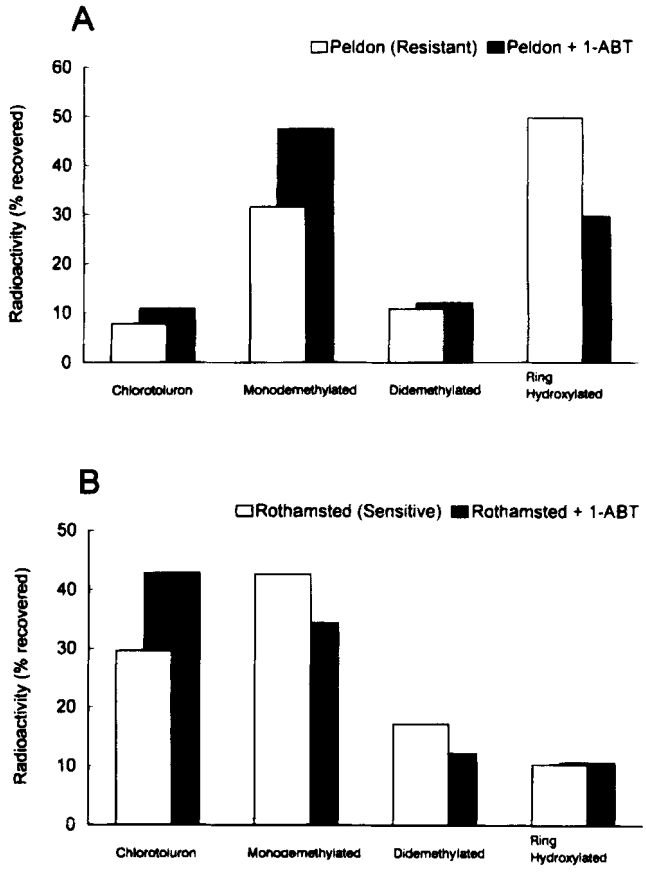


Fig. 4. Effect of 1-ABT on chlorotoluron metabolite distribution in (A) resistant and (B) susceptible *Alopecurus myosuroides*. The data are expressed as a percentage of the radioactivity recovered associated with identifiable compounds.

of the parent herbicide and accumulation of the non-hydrolysable conjugates (Table 1). Levels of demethylated metabolites increased in response to inhibitor treatment in the resistant biotype (Fig. 4A), probably reflecting a shift of metabolism into the demethylase pathway(s). When susceptible plant material was administered herbicide in the presence of 1-ABT, a different pattern was observed (Table 1; Fig. 4B). Considerably more (37%) parent chlorotoluron remained unmetabolised within the tissue, and the amount of non-hydrolysable conjugates decreased. However, in contrast to the results obtained with resistant plant material, accumulation of demethylated products decreased with susceptible material and accumulation of the ring methyl-hydroxylated metabolite was unaffected by 1-ABT treatment (Fig. 4B) in these plants. The reduced amounts of non-hydrolysable conjugates in the presence of 1-ABT in both resistant and susceptible biotypes indicate that this fraction is likely to derive from oxidised metabolites generated by the action of cytochrome P450 enzyme(s).

Ring methyl-hydroxylation of chlorotoluron was previously shown to occur in microsomal fractions of both wheat and maize.[14,21] This activity was supported by NADPH, and inhibited by carbon monoxide and 1-ABT, characteristic of reactions catalysed by cytochromes P450. We prepared microsomal fractions from both susceptible and resistant *A. myosuroides* biotypes. These preparations were incubated with radiolabelled chlorotoluron in the presence of NADPH, and subsequently solvent extracted. Analysis of these extracts by TLC did not reveal any detectable metabolites of the herbicide. The microsomal fractions were also examined for the presence of cytochrome P450 by carbon monoxide difference spectroscopy. The characteristic spectrum of the carbon monoxide adduct of reduced cytochrome P450 was not detected with preparations from either susceptible or resistant biotypes. It would appear that in membranes prepared from this species, cytochromes P450 are either present at levels lower than the detection level of this technique (*c.* 10 pmol cytochrome P450), or are sufficiently labile to be destroyed during microsome preparation.

4 CONCLUSIONS

The evidence obtained from metabolite analysis of susceptible and resistant *A. myosuroides* biotypes indicates that both biotypes possess the ability to metabolise the herbicide chlorotoluron. The difference between the two biotypes lies in the extent of metabolism and the spectrum of metabolites generated. In both biotypes, ring methyl-hydroxylation is likely to be the most important pathway leading to detoxification, as was shown in tolerant wheat and maize.[9,12,14,21] Inhibition of this activity by 1-ABT was observed in the Peldon biotype.

The metabolites generated from chlorotoluron in both biotypes are characteristic of the action of cytochrome P-450 monooxygenase(s), and inhibition of ring methyl-hydroxylation by 1-ABT in the Peldon biotype provides further evidence that this activity is likely to be mediated by a cytochrome P450. The data support the hypothesis that either the expression of a ring methyl-hydroxylase, sensitive to inhibition by 1-ABT, is markedly increased in the resistant (Peldon) biotype in comparison to the susceptible biotype, or that a novel ABT-sensitive ring methyl-hydroxylase, not found in the susceptible biotype, is present in Peldon plants. No evidence for the presence of cytochrome P-450 enzymes in microsomal fractions of either resistant or susceptible biotypes of *A. myosuroides* was obtained in this study, however. This is most likely due to a combination of low endogenous levels of these enzymes with the well-known instability of plant cytochromes P450 during isolation.[11,12]

In resistant (Peldon) tissue, simultaneous treatment with chlorotoluron and 1-ABT increased the proportion of parent herbicide and the relatively phytotoxic monodemethylated metabolite from 18% to 28%. Although the latter proportion is considerably lower than that found in the non-ABT treated susceptible biotype (53·4%), the increase in the presence of 1-ABT in the Peldon biotype must be sufficient to account for the previously demonstrated synergistic effect of 1-ABT on chlorotoluron treatment of resistant *A. myosuroides* at the whole plant level.[17]

In conclusion, it is perhaps not surprising that Gramineae species other than cereal crops have been shown to possess the ability to metabolize herbicides whose selectivity is based on metabolism. It would appear from the data presented here that only a relatively minor quantitative change in the ability of previously susceptible *A. myosuroides* to metabolise the herbicide chlorotoluron has resulted in a loss of selectivity of this herbicide. This or similar changes have possibly resulted in metabolism-based cross-resistance to other herbicide classes.

ACKNOWLEDGEMENTS

The work described was supported by a Biotechnology and Biological Sciences Research Council (BBSRC)-CASE studentship award in collaboration with IACR-Rothamsted. IACR receives grant-aided support from the BBSRC.

The authors would like to thank S. R. Moss (Crop Management Department, IACR-Rothamsted) for his generous supply of *A. myosuroides* seed, G. Foxon (Zeneca Agrochemicals, Jealotts Hill, UK) for supply of labelled chlorotoluron, W. J. Owen (Biochemistry Department, Royal Holloway) for gifts of the reference

metabolites and C. Edwards (Physiology and Biochemistry Department, IACR-Rothamsted) for assisting in the identification of metabolites.

REFERENCES

1. LeBaron, H. M. & McFarland, J., Herbicide resistance in weeds and crops: an overview and prognosis. In *Managing Resistance to Agrochemicals: from Fundamental Research to Practical Strategies*, ed. M. B. Green, H. M. LeBaron & W. K. Moberg. ACS Symposium Series 412, Washington, 1990, pp. 336–53.
2. Moss, S. R., Herbicide resistance in blackgrass (*Alopecurus myosuroides*). *Brit. Crop Prot. Conf.—Weeds* (1987) 879–86.
3. Moss, S. R. & Orson, J. H., The distribution of herbicide-resistant *Alopecurus myosuroides* (black-grass) in England. *Asp. Appl. Biol.*, **18** (1988) 177–85.
4. Moss, S. R., Herbicide cross-resistance in slender foxtail (*Alopecurus myosuroides*). *Weed Sci.*, **38** (1990) 492–6.
5. Kemp, M. S. & Caseley, J. C., Synergistic effects of 1-aminobenzotriazole on the phytotoxicity of chlorotoluron and isoproturon in a resistant population of blackgrass (*Alopecurus myosuroides*). *Brit. Crop Prot. Conf.—Weeds* (1987) 895–9.
6. Hall, L. M., Holtum, J. A. M. & Powles, S. B., Mechanisms responsible for cross resistance and multiple resistance. In *Herbicide Resistance in Plants—Biology and Biochemistry*, ed. S. B. Powles & J. A. M. Holtum. CRC Press, Boca Raton, 1994, pp. 243–61.
7. Ryan, P. J. & Owen, W. J., The mechanism of selectivity of chlorotoluron between cereals and grassweeds. *Brit. Crop Prot. Conf.—Weeds* (1982) 317–24.
8. Cabanne, F., Gaillardon, P. & Scalla, R., Phytotoxicity and metabolism of chlorotoluron in two wheat varieties. *Pestic. Biochem. Physiol.*, **23** (1985) 212–20.
9. Gonneau, M., Pasquette, B., Cabanne, F. & Scalla, R., Metabolism of chlorotoluron in tolerant species: possible role of cytochrome P-450 monooxygenases. *Weed Res.*, **28** (1988) 19–25.
10. O'Keefe, D. P., Lenstra, R. & Omer, C. A., An enzymatic basis for herbicide resistance: cytochrome P-450 monooxygenases. In *Plant Protein Engineering*, ed. P. R. Shewry & S. Gutteridge. Edward Arnold, London, 1991, pp. 281–91.
11. Bolwell, G. P., Bozak, K. & Zimmerlin, A., Plant cytochrome P450. *Phytochemistry*, **37** (1994) 1491–506.
12. Gaillardon, P., Cabanne, R., Scalla, R. &. Durst, F., Effect of mixed function oxidase inhibitors on the toxicity of chlorotoluron and isoproturon to wheat. *Weed Res.*, **25** (1985) 397–402.
13. Burnet, M. W. M., Loveys, B. R., Holtum, J. A. M. & Powles, S. B., A mechanism of chlorotoluron resistance in *Lolium rigidum*. *Planta*, **190** (1993) 182–89.
14. Fonne-Pfister, R. & Kreuz, K., Ring methyl-hydroxylation of chlorotoluron by an inducible cytochrome P450-dependent enzyme from maize. *Phytochemistry*, **29** (1990) 2793–96.
15. Mougin, C., Cabanne, F. & Scalla, R., Additional observations on the chlorotoluron hydroxylase and *N*-demethylase activities in wheat microsomes. *Plant Physiol. Biochem.*, **30** (1992) 769–78.
16. Ryan, P. J., Gross, D., Owen, W. J. & Laanio, T. L., The metabolism of chlorotoluron, diuron and CGA 43 057 in tolerant and susceptible plants. *Pestic. Biochem. Physiol.*, **16** (1981) 213–21.
17. Kemp, M. S. & Caseley, J. C., Synergists to combat herbicide resistance. In *Herbicide Resistance in Weeds and Crops*, ed. J. C. Caseley, G. W. Cussans & R. K. Atkin. Butterworths-Heineman, Oxford, 1991, pp. 279–92.
18. Burnet, M. W. M., Hilderbrand, O. B., Holtum, J. A. M. & Powles, S. B., Amitrole, triazine, substituted urea, and metribuzin resistance in a biotype of rigid ryegrass (*Lolium rigidum*). *Weed Sci.*, **39** (1991) 317–23.
19. Hallahan, D. L., Dawson, G. W., West, J. M. & Wallsgrove, R. M., Cytochrome P-450 catalysed monoterpene hydroxylation in *Nepeta mussinii*. *Plant Physiol. Biochem.*, **30** (1992) 435–43.
20. Canivec, M.-C., Cagnac, B., Cabanne, F. & Scalla, R., Induced changes of chlorotoluron metabolism in wheat cell suspension cultures. *Plant Physiol. Biochem.* **27** (1989) 193–201.
21. Mougin, C., Cabanne, F., Canivec, M.-C., & Scalla, R., Hydroxylation and *N*-demethylation of chlorotoluron by wheat microsomal enzymes. *Plant Sci.*, **66** (1990) 195–203.
22. Hyde, R. J., Cheriton, A. K., Hallahan, D. L. & Bowyer, J. R., Herbicide resistance and cytochrome P-450. *Brit. Crop Prot. Conf.—Weeds* (1993) 549–54.